PLANTS HAVING MODIFIED REPRODUCTIVE CAPACITY

This invention relates to plants having modified reproductive capacity. In particular, it relates to a plant reproductive tissue specific promoter and its use in promoting transcription/expression of associated genes in plant reproductive tissue, including for the purpose of producing plants which have diminished reproductive capacity or which are sterile.

INTRODUCTION

It is desirable that the genetic basis of reproduction/flower development in plants be determined. Identification of genes involved in plant reproduction and/or flower development together with the regulatory elements which control their expression in reproductive tissue allows for modulation of the reproductive capacity of plants and specifically enables the production of reproductively null (sterile) plants. Identification of the regulatory elements involved further allows for the expression in reproductive tissue of genes which are heterologous to the plant where that is desirable.

The applicants have now identified and isolated such a reproductive tissue specific promoter which endogenously regulates expression of a peptide involved in the reproductive cycle of *Pinus radiata*. It is broadly towards this promoter, to its homologs in other plants and to its use in effecting expression of associated genes within the reproductive tissue of plants that the present invention is directed.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a polynucleotide which has a nucleotide sequence of from nucleotides 1 to 1320 of Figure 2 and which has the ability, when operatively associated with a nucleotide sequence encoding a peptide, to promote transcription of that nucleotide sequence, or a polynucleotide which is a functionally equivalent variant thereof.

In a second aspect, the invention provides a plant reproductive tissue promoter which has a nucleotide sequence of from nucleotides 1 to 1320 of Figure 2, or a functionally equivalent variant thereof.

In a further aspect, the invention provides a DNA construct which comprises:

(a) a polynucleotide having activity as a transcriptional promoter as described above;

(b) an open reading frame polynucleotide coding for a peptide; and

(c) a termination sequence.

In yet a further aspect, the invention provides a DNA construct which comprises:

(a) a promoter sequence as defined above;

(b) an open reading frame polynucleotide coding for a peptide; and

(c) a termination sequence.

In each construct the open reading frame can be in a sense orientation, or an anti-sense orientation.

In one embodiment, the open reading frame polynucleotide encodes a peptide having the sequence of Figure 1.

In other embodiments, the open reading frame polynucleotide encodes a peptide which, when expressed in reproductive tissue of a plant, causes said plant's reproductive organs to abort, to redefine themselves as vegetative, or to stop development.

In still another embodiment, the open reading frame polynucleotide encodes a peptide which, when expressed in the reproductive tissue of a plant, causes cell death.

In yet another embodiment, the open reading frame polynucleotide encodes a peptide, which when expressed in reproductive tissue of a flowering plant, causes an alteration in the timing of flowering of said plant.

In a preferred form, the construct further includes:

(d) a selection marker sequence.

In a further aspect, the invention provides a transgenic plant cell which includes a construct as described above.

By "transgenic" as used herein, the applicants mean containing non-endogenous genetic material.

In another aspect, the invention provides a transgenic plant which includes a construct as described above.

In still another aspect, the invention provides a transgenic plant which contains a polynucleotide having activity as a transcriptional promoter as described above or a reproductive tissue promoter as described above, which plant has a reduced reproductive capacity.

It is particularly preferred that the plant be sterile.

Conveniently, in said plant said polynucleotide or promoter is operatively associated with a nucleotide sequence encoding a RNAse.

The plant can be a coniferous plant, such as a coniferous plant of the *Pinus* genus, or a tree such as a member of the *Eucalyptus* genus.

It is particularly preferred that the transgenic plant be a member of a species selected from *Pinus radiata, Pinus taeda, Pinus elliotti, Pinus clausa, Pinus palustrus, Pinus echinata, Pinus ponderosa, Pinus jeffrey, Pinus resinosa, Pinus rigida, Pinus banksiana, Pinus serotina, Pinus strobus, Pinus monticola, Pinus lambertiana, Pinus*

virginiana, Pinus contorta, Pinus cariboea, Pinus pinaster, Pinus brutia, Pinus eldarica, Pinus coulteri, Pinus nigra, Pinus sylvestris, Pinus tecunumannii, Pinus keysia, Pinus oocarpa and *Pinus maxinumoii.*

DESCRIPTION OF THE DRAWINGS

While the invention is broadly as defined above, it will be appreciated by those persons skilled in the art that it is not limited thereto and that it further includes embodiments of which the following description provides examples. In addition, the invention will be better understood through reference to the accompanying drawings in which:

Figure 1 shows the amino acid sequence of the reproductive peptide PrAG1, together with the nucleotide sequence coding therefor;

Figure 2 shows the sequence of the PrAG1 promoter, which is the focus of the present invention, isolated from *Pinus radiata*;

Figure 3 is an RNA gel blot analysis of PrAG1 mRNA accumulation in *Pinus radiata* organs. Twenty μg of total RNA from various organs was electrophoresed, blotted onto nylon membranes, and hybridized with 3'-terminal fragment of PrAG1 cDNA. Total RNA was isolated from immature male cone(M), immature female cone(F), vegetative shoot (V)s, needle (N) and stem (S). The 26S and 18S rRNA was used as control (bottom);

Figure 4 is a DNA gel blot analysis of *Pinus radiata* genomic DNA hybridized with the 3' terminal region of PrAG1. 20 μg genomic DNA was digested with BamHI (BA) and Bgl II (BG) EcoRI(E), HindIII (H), XhoI(X);

Figure 5 is a Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis showing reproductive-organ specific expression of PrAG1. RT-PCR analysis was performed on total RNA isolated from different organs of radiata Pine: (M) immature male cone, (F) immature female cone, (Vs) vegetative shoot, (N) needle and (S) stem. mads box genes were amplified with PrAG1 gene-specific oligonucleotides. Products

from the PCR reactions were electrophoresed, blotted, and hybridized with a labelled probe of PrAG1 specific fragment;

Figure 6 shows the construction of pRAGPR;

Figure 7. PCR analysis of transgenic tobacco lines. Genomic DNA (200 ng each) from controls and putative transgenic tobacco plants was used as template along with primers for NPTII gene. Lane PC is positive control (20 ng pRAGPR plasmid was used as a template); C1-C3 are control nontransformed tobacco plants; lanes 1a, 1b, 2, 3a, 3b, 3c, 4a, 4b are transgenic plants. The size of NPT II gene PCR product is indicated on the right;

Figure 8. Southern analysis of transgenic and control tobacco lines to confirm integration of PrAG1promoter-RNAse gene cassette. Genomic DNA (20 ug each) was digested with Hind III and Sac I enzymes, electrophoresed and transferred to a nylon membrane. ^{32}-P labeled RNAse gene was used as a probe. The size of PrAG1promoter-RNAse gene cassette is indicated on the right. Designation of control and transgenic plants is as indicated in Figure 7; and

Figure 9. Southern analysis of transgenic and control tobacco lines to confirm integration profiles of PrAG1promoter-RNAse gene cassette. Genomic DNA (20 ug each) was digested with Hind III enzyme, electrophoresed and transferred to a nylon membrane. ^{32}-P labeled RNAse gene was used as a probe. Sizes of DNA fragments hybridizing to the RNAse gene probe are indicated on the right. Designation of control and transgenic plants is as indicated in Figure 7.

DESCRIPTION OF THE INVENTION

As broadly outlined above, the applicants have identified a plant promoter which is involved in plant reproduction. The promoter, which was isolated from *Pinus radiata* is called herein the "PrAG1 promoter".

The nucleotide sequence of the PrAG1 promoter is given in Figure 2 from nucleotides 1 to 1320. It will however be appreciated that the invention is not

restricted only to the polynucleotide having that specific nucleotide sequence. Instead, the invention also extends to functionally equivalent variants of that polynucleotide.

The phrase "functionally equivalent variants" recognises that it is possible to vary nucleotide sequence while retaining substantially equivalent functionality.

Variants can have a greater or lesser degree of homology as between the variant nucleotide sequence and the original.

Polynucleotide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another sequence, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The BLASTN software are available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu/pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is also preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in W. R. Pearson and D. J. Lipman, "Improved Tools for Biological Sequence Analysis", *Proc. Natl. Acad. Sci. USA* 85:2444-2448 (1988).

The following running parameters are preferred for determination of alignments and similarities using BLASTN that contribute to E values (as discussed below) and percentage identity: Unix running command: blastall -p blastn -d embldb -e 10 -G 1 -E 1 -r 2 -v 50 -b 50 -I queryseq -o results; and parameter default values:

-p Program Name [String]

-d Database [String]

-e Expectation value (E) [Real]

-G Cost to open a gap (zero invokes default behaviour) [Integer]

-E Cost to extend a cap (zero invokes default behaviour) [Integer]

-r Reward for a nucleotide match (blastn only) [Integer]

-v Number of one-line descriptions (V) [Integer]

-b Number of alignments to show (B) [Integer]

-i Query File [File In]

-o BLAST report Output File [File Out] Optional

The "hits" to one or more database sequences by a queried sequence produced by BLASTN, FASTA, or a similar algorithm, align and identify similar portions of sequences. The hits are arranged in order of the degree of similarity and the length of sequence overlap. Hits to a database sequence generally represent an overlap over only a fraction of the sequence length of the queried sequence.

The BLASTN and FASTA algorithms also produce "Expect" or E values for alignments. The E value indicates the number of hits one can "expect" to see over a certain number of contiguous sequences by chance when searching a database of a certain size. The Expect value is used as a significance threshold for determining whether the hit to a database, such as the preferred EMBL database, indicates true similarity. For example, an E value of 0.1 assigned to a hit is interpreted as meaning that in a database of the size of the EMBL database, one might expect to see 0.1 matches over the aligned portion of the sequence with a similar score simply by chance. By this criterion, the aligned and matched portions of the sequences then have a 90% probability of being the same. For sequences having an E value of 0.01 or less over aligned and matched portions, the probability of finding a match by chance in the EMBL database is 1% or less using the BLASTN or FASTA algorithm.

It is also recognised that as the function of the polynucleotide is as a transcriptional promoter there are regions of the polynucleotide which are more critical to, and characteristic of, this function than others. An example are the TATA boxes at positions 280 to 286, 282 to 288 and 1015 to 1021 from the 5' end of the sequence. Therefore, polynucleotides which include these regions of the polynucleotide of Figure 2 and have equivalent transcriptional functionality are c ntemplated

variants, even where there is a lesser degree of homology elsewhere in the sequence.

According to one embodiment, "variant" polynucleotides, with reference to the polynucleotide of the present invention, preferably comprise sequences having the same number or fewer nucleic acids than the polynucleotide of the present invention and producing an E value of 0.01 or less when compared to the polynucleotide of the present invention. That is, a variant polynucleotide is any sequence that has at least a 99% probability of being the same as the polynucleotide of the present invention, measured as having an E value of 0.01 or less using the BLASTN or FASTA algorithms set at the parameters discussed above.

Variant polynucleotide sequences will also generally hybridize to the recited polynucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65°C.

It is of course expressly contemplated that homologs to the PrAG1 promoter exist in other plants, particularly other coniferous plants, including other members of the *Pinus* genus. Such homologs are also "functionally equivalent variants" of PrAG1 promoter as the phrase is used herein.

DNA sequences from plants other than *Pinus radiata* which are homologs of the PrAG1 promoter may be isolated by high throughput sequencing of cDNA libraries prepared from such plants. Alternatively, oligonucleotide probes based on the sequence for the PrAG1 promoter provided in Figure 2 can be synthesized and used to identify positive clones in either cDNA or genomic DNA libraries from other plants by means of hybridization or PCR techniques. Probes should be at least about 10, preferably at least about 15 and most preferably at least about 20 nucleotides in length. Hybridization and PCR techniques suitable for use with such ligonucleotide probes are well known in the art. Positive clones may be analyzed by restriction enzyme digestion, DNA sequencing or the like.

The polynucleotides of the present invention may be generated by synthetic means using techniques well known in the art. Equipment for automated synthesis of oligonucleotides is commercially available from suppliers such as Perkin Elmer/Applied Biosystems Division (Foster City, CA) and may be operated according to the manufacturer's instructions.

The PrAG1 promoter of the invention is plant reproductive-tissue-specific. The primary importance of identification of the polynucleotide of the invention is therefore that it enables the reproductive capacity of plants to be modulated. This modulation will generally involve a reduction in the reproductive capacity of the plant.

Any conventional technique for effecting this can be employed. Examples include co-suppression or anti-sense strategies, a dominant negative approach, or techniques which involve expressing enzymes (such as RNAses) to digest, or otherwise be lethal to, RNA post-transcription of a target gene.

Co-suppression can be effected in a manner similar to that discussed, for example, by Napoli *et al* (Plant Cell 2:279-290, 1990) and de Carvalho Niebel *et al* (Plant Cell 7:347-258, 1995). In some cases, it can involve overexpression of the gene of interest through use of multiple constructs of the PrAG1 promoter and gene to be suppressed.

Anti-sense strategies involve using the PrAG1 promoter to effect expression or transcription of DNA with the expression/transcription product being capable of interfering with translation of mRNA transcribed from the gene to be suppressed. This will normally be through the expression/transcription product hybridising to and forming a duplex with the target mRNA.

The expression/transcription product can be a relatively small molecule and still be capable of disrupting mRNA translation. However, the same result is achieved by expressing the target gene in an anti-sense orientation such that the RNA produced

by transcription of the anti-sense oriented gene is complementary to all or part of the endogenous target mRNA.

Such anti-sense strategies are described generally by Robinson-Benion *et al.*, (1995), Anti-sense techniques, *Methods in Enzymol.* 254(23):363-375 and Kawasaki *et al.*, (1996), *Artific. Organs* 20 (8): 836-848.

Dominant negative approaches involve using the PrAG1 promoter to effect the expression of a modified DNA binding/activating protein which includes a DNA binding domain but not a activator domain. The result is that the protein binds to DNA as intended but fails to activate, while at the same time blocking the binding of the DNA binding/activating peptides which normally bind to the same site.

It is however presently preferred that the reproductive capacity of the plant be reduced or eliminated through the use of the PrAG1 promoter to drive transcription and expression of a nucleotide sequence which encodes an RNAse within the plant reproductive tissue. Such an approach, in which the PrAG1 promoter is coupled to the RNAse, RNS2, is exemplified herein.

To give effect to the above strategies, the invention also provides DNA constructs. The constructs include the PrAG1 promoter sequence, the DNA intended to be transcribed/expressed (such as the PrAG1 gene in sense or in anti-sense orientation or a polynucleotide encoding an RNAse) and a termination sequence, operably linked to the DNA sequence to be transcribed. The promoter sequence is generally positioned at the 5' end of the DNA sequence to be transcribed, and is employed to initiate transcription of the DNA sequence.

The DNA with which the PrAG1 promoter is operatively associated can encode any peptide it is desirable to express in plant reproductive tissue. As indicated above, this includes the peptide encoded by PrAG1, but can also be another peptide. That other peptide can be a peptide which, when produced, causes the reproductive organs of the plant to abort, redefine themselves as vegetative or stop development. The peptide encoded can, for example, also be a peptide causing cell death. Illustrative peptides/genes are Diphtheria Toxin A (DTA), Barnase (from Bacillus

amyloliquefaciens), apoptosis genes, glucanase, and RNAses, with the selection of each being a matter of choice for the art skilled worker.

Alternatively, the peptide which is to be expressed under the control of the PrAG1 promoter can be one which, when produced, alters the timing of flowering (ie. either delays or accelerates flowering, such as the ELF-3 and CONSTANS flowering time genes).

The peptide to be expressed can be ligated to the promoter in a sense or antisense orientation, dependant upon the desired effect.

The termination sequence, which is located 3' to the DNA sequence to be transcribed, may come from the same gene as the PrAG1 promoter or may be from a different gene. Many termination sequences known in the art may be usefully employed in the present invention, such as the 3' end of the *Agrobacterium tumefaciens* nopaline synthase gene. However, preferred termination sequences are those from the original gene or from the target species to be transformed.

The DNA constructs of the present invention may also contain a selection marker that is effective in plant cells, to allow for the detection of transformed cells containing the construct. Such markers, which are well known in the art, typically confer resistance to one or more toxins. One example of such a marker is the NPTII gene whose expression results in resistance to kanamycin or hygromycin, antibiotics which are usually toxic to plant cells at a moderate concentration (Rogers *et al.*, in *Methods for Plant Molecular Biology*, A Weissbach and H Weissbach eds, Academic Press Inc., San Diego, CA (1988)). Other examples of markers include visible selection markers such as Green Fluorescent Protein (GFP) and herbicide resistance genes. Alternatively, the presence of the desired construct in transformed cells can be determined without reference to marker genes, by means of other techniques well known in the art, such as Southern and Western blots.

Techniques for operatively linking the components of the DNA constructs are well known in the art and include the use of synthetic linkers containing one or more restriction endonuclease sites as described, for example, by Maniatis *et al.*,

(*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratories, Cold Spring Harbour, NY, 1989). The DNA construct may be linked to a vector having at least one replication system, for example, *E. coli*, whereby after each manipulation, the resulting construct can be cloned and sequenced and the correctness of the manipulation determined.

The DNA constructs of the present invention may be used to transform a variety of plants. In one embodiment, these will be plants of the *Pinus* genus. In a preferred embodiment, the DNA constructs are employed to transform *Pinus radiata, Pinus taeda, Pinus elliotti, Pinus clausa, Pinus palustrus, Pinus echinata, Pinus ponderosa, Pinus jeffrey, Pinus resinosa, Pinus rigida, Pinus banksiana, Pinus serotina, Pinus strobus, Pinus monticola, Pinus lambertiana, Pinus virginiana, Pinus contorta, Pinus cariboea, Pinus pinaster, Pinus brutia, Pinus eldarica, Pinus coulteri, Pinus nigra, Pinus sylvestris, Pinus tecunumannii, Pinus keysia, Pinus oocarpa* and *Pinus maxinumoii*; and hybrids between the above species.

The constructs can also be used to transform other plants such as trees selected from *Abies amabilis, Abies balsamea, Abies concolor, Abies grandis, Abies lasiocarpa, Abies magnifica, Abies procera, Chamaecyparis lawsoniona, Chamaecyparis nootkatensis, Chamaecyparis thyoides, Huniperus virginiana, Larix decidua, Larix laricina, Larix leptolepis, Larix occidentalis, Larix siberica, Libocedrus decurrens, Picea abies, Picea engelmanni, Picea glauca, Picea mariana, Picea pungens, Picea rubens, Picea sitchensis, Pseudotsuga menziesii, Sequoia gigantea, Sequoiasempervirens, Taxodium distichum, Tsuga canadensis, Tsuga heterophylla, Tsuga mertensiana, Thuja occidentalis, Thuja plicata;* Eucalypts, such as *Eucalyptus alba, Eucalyptus bancroftii, Eucalyptus botyroides, Eucalyptus bridgesiana, Eucalyptus calophylla, Eucalyptus camaldulensis, Eucalyptus citriodora, Eucalyptus cladocalyx, Eucalyptus coccifera, Eucalyptus curtisii, Eucalyptus dalrympleana, Eucalyptus deglupta, Eucalyptus delagatensis, Eucalyptus diversicolor, Eucalyptus dunnii, Eucalyptus ficifolia, Eucalyptus globulus, Eucalyptus gomphocephala, Eucalyptus gunnii, Eucalyptus henryi, Eucalyptus laevopinea, Eucalyptus macarthurii, Eucalyptus macrorhyncha, Eucalyptus maculata, Eucalyptus marginata, Eucalyptus megacarpa, Eucalyptus melliodora, Eucalyptus nicholii, Eucalyptus nitens, Eucalyptus nova-anglica, Eucalyptus obliqua, Eucalyptus obtusiflora, Eucalyptus oreades, Eucalyptus pauciflora, Eucalyptus polybractea, Eucalyptus regnans, Eucalyptus resinifera,*

Eucalyptus robusta, Eucalyptus rudis, Eucalyptus saligna, Eucalyptus sideroxylon, Eucalyptus stuartiana, Eucalyptus tereticornis, Eucalyptus torelliana, Eucalyptus urnigera, Eucalyptus urophylla, Eucalyptus viminalis, Eucalyptus viridis, Eucalyptus wandoo and *Eucalyptus youmanni*; and hybrids between any of the above species.

As discussed above, transformation of a plant with a DNA construct including an open reading frame coding for a peptide wherein the open reading frame is orientated in a sense direction can, in some cases, lead to a decrease in expression of the peptide by co-suppression. Transformation of the plant with a DNA construct comprising an open reading frame in an anti-sense orientation or a non-coding (untranslated) region of a gene will lead to a decrease in the expression of the peptide in the transformed plant.

Techniques for stably incorporating DNA constructs into the genome of target plants are well known in the art and include *Agrobacterium tumefaciens* mediated introduction, electroporation, protoplast fusion, injection into reproductive organs, injection into immature embryos, high velocity projectile introduction and the like. The choice of technique will depend upon the target plant to be transformed.

Once the cells are transformed, cells having the DNA construct incorporated into their genome may be selected by means of a marker, such as the NPT II or kanamycin resistance markers discussed above. Transgenic cells may then be cultured in an appropriate medium to regenerate whole plants, using techniques well known in the art. In the case of protoplasts, the cell wall is allowed to reform under appropriate osmotic conditions. In the case of seeds or embryos, an appropriate germination or callus initation medium is employed. For explants, an appropriate regeneration medium is used.

For a review of regeneration of forest trees such as those of the *Pinus* genus, see Dunstan *et al.*, Somatic embryogenesis in woody plants. In: Thorpe, T.A. ed. 1995: *in vitro* embryogenesis of plants. Vol 20 in Current Plant Science and Biotechnology in Agriculture, Chapter 12, pp. 471-540.

The promoter, and constructs containing it, are not restricted in use to plants of forestry species. They can also be used to transform other agronomically important plants in which modulation of reproductive capacity (particularly the timing and abundance of flowering) is desirable. Such plants include cereals, rice, maize, wheat, barley, oats, rye, soyabean and canola.

The resulting transformed plants may be reproduced sexually or asexually, using methods well known in the art, to give successive generations of transgenic plants.

The invention will now be described with reference to the following non-limiting examples.

EXPERIMENTAL

SECTION 1

1. PrAG1 cDNA Cloning:

Total RNA was purified from immature cone of radiata pine according to the protocol of Charles Ainsworth (Plant Molecular Biology Reporter, 12(3), 1994: 198-203). The mRNA was isolated with oligo-T cellulose column. With mRNA as template, the cDNA was synthesised with Cap-Finder cDNA synthesis kit (ClonTech Co.). The cDNA was inserted into Lambda TriplEx Vector (ClonTech Co.), then packaged it with Gigapack@ III packaging extracts (Stratagene Co.) to obtain a cDNA library.

Two degenerate primers were designed:

3' PCR primer: 5' GCIGTIAGIYCITCICCCAT3' ; (SEQ ID NO. 6)
5' PCR primer: 5' AAYCGICARGTIACITT3' (SEQ ID NO. 7)

These primers were used to perform RT-PCR based differential screening on RNA from various female tissue sources, including immature female buds, vegetative tissue from needles and later stages of development. The 50-ul reaction mixture contained 2.5 Units Taq DNA polymerase, 1X Polymerization Buffer (both from

ClonTech Co.), 1mMMgCl$_2$, 0.2mMdNTP and 0.25uM primers. The PCR was performed under the following conditions: denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1 min for 30 cycles on a Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT, USA).

Fragments were obtained mostly from immature female bud tissue RNA samples. Several DNA fragments were cloned into pGEM-T vector and sequenced. Sequence analysis showed that most of these fragments contained similar sequences. One of the DNA fragments (309 bp) was chosen. This was used as a probe to screen the cDNA library to clone its full-length cDNA and resulted in the cDNA clone, PrAG1. The sequence of PrAG1 was analysed on both strands by the Sanger's dideoxy method (Sanger *et al.*, 1977, Proc. Natal. Acad. Sci. U.S.A. 74: using a Sequenase kit (United States Biochemical co.).

The resulting sequence is shown in Figure 1 gives the nucleotide sequence coding for the peptide of the invention together with the predicted amino acid sequence.

Sequence comparison and phylogenetic analysis were conducted with the software program MacDNASIS (Version 3.5, Hitachi Corp.). The results of analysis revealed PrAG1 to be a MADS box gene.

2. PrAG1 Promoter Cloning:

I. Genomic DNA purification:

Genomic DNA was purified from young needles according to a CTAB method as described below.

1) 2 g of young needles of *Pinus* radiata were ground in liquid nitrogen (mortar and pestle) to a fine powder.

2) This powder was mixed with 15 mL of pre-warmed CTAB extraction buffer [3% CTAB(W/V), 100mM Tris-HCl pH8.0, 20 mM EDTA pH8.0, 1.4 M NaCl, 1% PVP 940,000, 1% beta mercaptoethanol] and incubated at 65°C for one hour.

3) To the above mixture 15 mL chloroform was added and mixed gently.

4) The contents were centrifuged at 10,000g for 20 minutes at 4°C.

5) The supernatant was transferred to a new tube, and mixed with 1/10 volume of 3M sodium acetate (pH4.8), and 0.7 volume of isopropanol. The DNA was precipitated at -20°C for 30 minutes.

6) The DNA was pelleted at 10,000 g for 10 minutes at 4°C.

7) The DNA pellet was then air dried and resuspended in 2 mL TE buffer (10 mM Tris-HCl pH7.5, 1mM EDTA pH8.0) and 2 µl of RNAse A (10µg/µL) was added. The contents were incubated at 37°C for 30 minutes to remove any RNA from the sample.

8) After the incubation, 2 mL of 5M Ammonium acetate and 10 mL of 100% ethanol were added and the contents kept at -20°C for 15 minutes.

9) The mixture was then centrifuged at 10,000 g for 10 minutes at 4°C to pellet DNA. The DNA pellet was washed in 70% ethanol twice.

10) The DNA pellet was air dried and resuspended in 200 uL TE bufffer.

II. Cloning of PrAG1 Promoter with Two step Genomic DNA Walking.

1) The Universal Genome Walker Kit (CLONTECH) was employed. For the first step genomic DNA walking, two PrAG1 specific primers were designed and synthesized according to the PrAG1 cDNA sequence. The sequences of the primers were:

Primer GSP1: 5' CGC CTT CTT CAA TAA ACC ATT TCG GCG CTT 3' SEQ ID NO. 8
Primer GSP2: 5' GAC CTG TCG GTT CGT AGT ATT TTC AAT CCT 3' SEQ ID NO. 9

2) Based upon the promoter sequence we got from step 1), two PrAG1 promoter sequence specific primer were designed and synthesized. The primers were:

Primer GSP3: 5' TTC GTC CTC CAT TTT GTG CGC TCT CCA TTC 3' SEQ ID NO. 10
Primer GSP4: 5' GCA CTC CAC TCT TCC TTT ATT TCT TAC CAC 3'. SEQ ID NO. 11

3) According to the User Manual of Universal Genome Walker Kit, 13 genome walker libraries were constructed after genomic DNA digestion with restriction enzymes: EcoR V, Sca I, Dra I, Pvu II, Ssp I, Stu I, Sma I, Hap I, BsaB I, Bcl136 II, Pml I, Nru I, Hic II.

4) With 13 genome walker libraries as templates, and adaptor primer 1 (AP1 primer from kit) and GSP1 primer, first round PCR was performed under the conditions suggested by the kit manufacturer. After agarose electrophoresis analysis of the PCR product, second round PCR was performed with the nested primers AP2 (Adaptor primer from the kit) and GSP2. The PCR products from the second round PCR were purified and cloned into pGEM-T easy vector (Promega). Following sequence analysis, and DNA sequence comparison with PrAG1 cDNA, one DNA fragment of 1105 bp from Sca I genome walker library was obtained which was identified as the promoter region of PrAG1, based upon the overlapped region between it and PrAG1 cDNA.

5) The second step genome walking was done with primer pair AP1 and GSP3, and primer pair AP2 and GSG4. A DNA fragment of 449 bp from the Dra I genome walker library was identified as the upstream sequence of the PrAG1 promoter cloned from the first step genome walking based on the sequence comparison of overlapped region between them.

6) The 1105bp and 449 bp fragments were used in PCR mediated DNA splicing to synthesize one continuous 1458 bp promoter fragment of PrAG1. This was done as described. One primer was synthesized based on the 5'end sequence of 1105 bp promoter fragment: Primer PLi, 5' AGT TAC TTA ACA ATG CGC AAC CAA GGC 3' (SEQ ID NO. 12). Primer pair PLi and GSP2 was used in PCR to get the promoter fragment of 1105 bp, in which the AP2 primer sequence was removed. This 1105 bp

fragment and 449 bp fragment was then added in one PCR tube as a template with the primer pair of AP2 and GAP2 to do the second round PCR to get the 1458 bp PCR fragment. The conditions of second round PCR were as follows: the first cycle at 95°C for 5 minutes, and 68°C for 10 min; the second cycle at 94°C for 30 seconds (DNA denaturing), DNA annealing at 60 C for 1 min, and DNA synthesis at 72 C for 2 minutes; this regime was cycled 30 times. This 1458 bp fragment was then cloned into pGEM-T easy vector (Promega) and subjected to DNA sequencing on both strands to confirm the DNA sequence and to make sure that no base changes occurred during the PCR process.

The sequence of the promoter, (which is the PrAG1 promoter), is given in Figure 2 from nucleotides 1 to 1320.

7) DNA sequence analysis has indicated that compared to its orthologs from other plants, the PrAG1 showed that there were two possible positions for transcription initiation: at position 791 or 1326 in the Figure 2 sequence. It was found three typical TATA boxes in the PrAG1 promoter at the position of 280 to 286, 282 to 288, 1015 to1021. Based on the start codon position and short 5' untranslated region in the PrAG1 cDNA, the transcription initiation point is identified as position 1326 in the Figure 2 sequence.

3. DNA and RNA Gel Blot Hybridizations:

Genomic DNA and RNA gel blots were made using standard techniques (Sambrook et al., 1989. Molecular Cloning : A Laboratory Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

RNA: Total RNA was prepared from needle, vegetative shoot, stem, immature female cone and immature male cone samples as described above. Briefly, 20µg of total RNA was denatured in formaldehyde loading buffer and fractionated by denaturing agarose gel electrophoresis on a formaldehyde containing gel. The agarose gel was stained with ethidium bromide and a picture taken as control. The RNA was then transferred to a nylon membrane by the capillary blotting method. The RNA was immobilised on the membrane by UV cross-linking and was prehybridized at 65°C

for 2 hours prior to hybridization in 0.5M Na-phosphate, pH 7.2, 7.5%SDS, 1mM EDTA, 100ug/mL salmon sperm DNA. A DNA fragment of PrAG1 3' end region was labelled with ^{32}P- dCTP (Decaprime II kit, Ambion, Austin, TX), and hybridised to the RNA blot overnight at 65°C. The blot was washed twice in 40mMNa-PO4, 1%SDS and 1mM EDTA for 30 minutes each at 65°C, and exposed to X-ray film with intensifying screens at –80°C.

DNA: Genomic DNA was prepared from needle tissue with CTAB method. Twenty μg genomic DNA was digested by Bam HI, Bgl II, Eco RI, Hind III and Xba I respectively. After agarose gel running, alkali blotting of DNA to Hybond N membranes was performed as described by the manufacturer (Amersham). The probe hybridisation and washing was as described for the RNA blotting analysis.

The results are shown in Figures 3 and 4.

4. RT-PCR:

Analysis was performed on total RNA isolated from needle, stem, vegetative shoot, immature female cone and immature male cone samples as described above. RNA was reverse-transcribed with MMLV reverse-transcriptase (Gibco BRL) according to the manufacturer's instructions. PCR was performed with two primers: 5'PCR primer (5' TTGTGTACAAATCATGGG3') (SEQ ID NO. 13) and 3'PCR primer (5' GTAAGCCCGTCACCCATC3') (SEQ ID NO. 14). Verification of the specificity of the PCR reactions was achieved through the use of controls that included amplification reaction with single primers, RNAse treatment of template, and no template. In those reactions in which no PCR product was detected, the quality of the RNA was tested by UV scanning, and agarose gel electrophoresis. ss-cDNA from the RT reaction was used as a template. The 50-ul reaction mixture contained 2.5 U Taq DNA polymease, 1X Polymerization Buffer (both from ClonTech Co.), 1mM $MgCl_2$, 0.2mM dNTP and 0.25μM primers. The PCR was performed under following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min for 30 cycles on Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT, USA). The PCR products were subjected to electrophoresis in agarose gel, and hybridization as described above.

The results are shown in Figure 5.

Discussion

Northern blot hybridization and RT-PCR analysis showed that PrAG1 mRNA is accumulated specifically in the immature female cone and immature male cone; there is no expression detected in needle, stem, and vegetative shoot (Figures 3 and 5). This tissue distribution profile, when combined with the fact that PrAG1 contains a MADS box, verifies that PrAG1 is a reproductive gene in *Pinus radiata* and that the PrAG1 promoter is reproductive-tissue specific.

Southern blot analysis showed that PrAG1 gene exists as a single copy in the genome of *Pinus radiata* (Figure 4).

SECTION 2

Construction of binary vector pRAGPR, plant transformation and regeneration f transgenic tobacco plants

A DNA fragment containing the PrAG1 promoter (1.46 kb, sequence of Figure 2) operably fused to an RNAse gene (0.95 kb, RNS2, Taylor *et al. Proc Natl Acad Sci,* USA 90 (11), 5118-5122 (1993)) and containing Hind III and Sac I sites was gel purified and ligated into the Hind III/Sac I sites of binary vector pRD420, containing the NPTII gene for plant selection, (provided by Dr. R.S.S. Datla, PBI, Saskatoon, Canada) resulting in the construct pRAGPR (Figure 6). The construct was introduced into *Agrobacterium tumefaciens* (strain c58 MP90), and used to transform and regenerate *Nicotiana tabacum* var. *Xanthi* by the standard leaf disc transformation method (Horsch *et al.* (1985), A simple and general method for transferring genes into plants. *Science* 227, 1229-1231). Control lines were also generated through leaf disk method without the selection process. After kanamycin selection, the putative transgenic plantlets were rooted in the rooting medium containing kanamycin and then moved to pots containing the Metromix 350 potting mix. Potted plants were maintained under controlled conditions in a growth chamber with 16h photoperiod. The plants were grown through the full life cycle of

the tobacco until senescence and the flowering of the transgenic tobacco assessed relative to controls. Transgenic plants were identified further by PCR with template of genomic DNA and Southern blot analysis to confirm the integration of pRAGPR in transgenic tobacco plants.

P lymerase Chain Reaction Amplification

To check the genomic DNA integration of pRAGPR in the transgenic tobacco plants, gene-specific primers for the NPTII gene were employed. The primers used were NPTII-5' primer 5'-GAA CAA GAT GGA TTG CAC GC-3' (SEQ ID NO. 15) and NPTII-3' primer 5'-GAA GAA CTC GTC AAG AAG GC-3' (SEQ ID NO. 16). Genomic DNA from each of the control lines and transgenic tobacco lines were isolated from the leaf tissue using the Qiagen DNAeasy kit as per manufacturer's instructions. PCR reactions (50-µl final volume) were performed using 5 µl of template DNA. Samples were heated to 95°C for 4 minutes, followed by 35 cycles of 95°C for 45 seconds, 55°C for 30 seconds, and 73°C for 2 minutes, with a final extension step of 73°C for 5 minutes in PTC100 thermal cycler (MJ Research). Amplified DNA fragments were analyzed on a 0.8 agarose gel and visualized by staining with ethidium bromide.

DNA Gel Blot Hybridization

To confirm the genomic integration of PrAG1 promoter-RNAse gene cassette in transgenic plants and to determine the copy number, Southern analysis was performed. For Southern analysis, genomic DNA (20µg) was digested with appropriate restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel. Two sets of Southerns were performed, one with digesting the genomic DNA with Hind III and Sac I to drop the PrAG1-RNAse cassette and another with Hind III digest alone to test for integration profiles. Following depurination in 0.25 M HCl and denaturation in 0.5 M NaOH, 1.5 M NaCl, the DNA was blotted onto a nylon membrane. The RNAse gene-specific probe (which is the whole RNAse gene) was radioactively labelled using a random-primed DECAprime II DNA labeling kit (Ambion, Austin, Texas). Filters were hybridized at 65°C in a hybridization buffer containing 0.5 M $NaPO_4$ (pH 7.5), 7.5% SDS, and 1mM EDTA. All filters were washed finally at 68°C in 20 mM $NaPO_4$ (pH 7.5), 1% SDS. Filters were then subjected to autoradiography.

R sults and Conclusions

Transg nic T bacc plants expressing PrAG1 promoter controlled RNAs gene. Eight independent transgenic lines of tobacco (*N. tobaccum* var *Xanthi*) with the PrAG1-RNAse fusion construct were obtained together with three control lines processed through the same tissue culture propagation method but without the selection. PCR was done on the putative transgenic plantlets, after rooting in selection medium, to confirm the integration of the NPTII gene. All the eight lines were positive for the NPTII gene and the controls were negative (Figure 7). The transgenic plants were propagated in pots along with the controls.

Southern analysis was done on the eight transgenic lines and the three controls to confirm the integration and profiles of integration of PrAG1promoter-RNAse gene cassette. Double digestion with Hind III and Sac I followed by probing with RNAse gene probe indicated that all the eight transgenic plants had the PrAG1-RNAse cassette (Figure 8). The single digest with Hind III indicated that 5 of the transgenic lines had single integration and three others had two copies of the cassette.

Out of the 5 transgenic plants with single integration two profiles were found based on molecular weight size bands that hybridized to the RNAse probe. Three plants had the same size band hybridizing at ~ 4kb (#s 3a, 3 c, 4a) whereas, the two other plants had a band hybridizing at ~3kb range (#s 3b, 4b)(Figure 9).

Of the three transgenic plants that had two copies of the cassette, two had same profile with a band at 5 -6 kb and another at 4 kb hybridizing to the RNAse probe (#1a, 1b), whereas the other had two bands, one at 8 kb and another at 4 kb that hybridized to RNAse probe (#2) (Figure 9).

All the three controls were negative for hybridization with the RNAse probe (Figure 8 and Figure 9).

Reproductive sterility in transgenic tobacco caused by pRAGPR.

All the controls and transgenic lines were allowed to senesce (approximately 3 months). The controls flowered normally whereas none of the transgenic tobacco

containing the pRAGPR construct flowered. The plants with single copy PrAG1-RNAse cassette grew at the same rate as controls and senesced at the same time. The transgenic plants with two copies of pRAGPR were slower to grow, and matured late (#1a, 1b and 2).

All transgenic plants eventually died and none flowered.

Another phenotypic change observed was in transgenic line #2, which developed additional lateral branches near the top. This may be due to the lack of or decreased apical dominance in these plants. Thus, the inhibition of flowering using the PrAG1-RNAse cassette may have an added benefit to increase the biomass of the plant through increased branching if the growing conditions are not limiting.

INDUSTRIAL APPLICATION

In its primary aspect, the invention provides a new, reproductive-tissue-specific promoter. This promoter can be used in transforming a wide variety of plants. The promoter can also be used to drive expression of any gene which it is desirable to express in plant reproductive organs, including flowering time genes.

The invention also has application in modulating, and in particular reducing or eliminating reproductive capacity in plants including those of the *Pinus* genus and *Eucalyptus* genus. Such plants have utility in forestry.

The availability of reproductively null or sterile pine or eucalyptus trees has the additional advantage that it will be possible to introduce further exogenous genetic material into those trees without the risk that the material will be passed on to other trees.

Those persons skilled in the art will appreciate that the specific description provided is exemplary only, and that modifications and variations may be made without departing from the scope of the invention.